

## Mammalian Genes Coordinately Regulated by Growth Arrest Signals and DNA-Damaging Agents

ALBERT J. FORNACE, JR.,<sup>1\*</sup> DANIEL W. NEBERT,<sup>2</sup> M. CHRISTINE HOLLANDER,<sup>1</sup> JENNIFER D. LUETHY,<sup>3</sup>  
MATILDA PAPATHANASIOU,<sup>1</sup> JOSEPH FARGNOLI,<sup>3</sup> AND NIKKI J. HOLBROOK<sup>3</sup>

*Radiation Oncology Branch, National Cancer Institute,<sup>1</sup> and Laboratory of Developmental Pharmacology, National Institute of Child Health and Human Development,<sup>2</sup> Bethesda, Maryland 20892, and Laboratory of Molecular Genetics, Gerontology Research Center, National Institute on Aging, Baltimore, Maryland 21224<sup>3</sup>*

Received 30 March 1989/Accepted 6 July 1989

More than 20 different cDNA clones encoding DNA-damage-inducible transcripts in rodent cells have recently been isolated by hybridization subtraction (A. J. Fornace, Jr., I. Alamo, Jr., and M. C. Hollander, *Proc. Natl. Acad. Sci. USA* 85:8800-8804, 1988). In most cells, one effect of DNA damage is the transient inhibition of DNA synthesis and cell growth. We now show that five of our clones encode transcripts that are increased by other growth cessation signals: growth arrest by serum reduction, medium depletion, contact inhibition, or a 24-h exposure to hydroxyurea. The genes coding for these transcripts have been designated *gadd* (growth arrest and DNA damage inducible). Two of the *gadd* cDNA clones were found to hybridize at high stringency to transcripts from human cells that were induced after growth cessation signals or treatment with DNA-damaging agents, which indicates that these responses have been conserved during mammalian evolution. In contrast to results with growth-arrested cells that still had the capacity to grow after removal of the growth arrest conditions, no induction occurred in HL60 cells when growth arrest was produced by terminal differentiation, indicating that only certain kinds of growth cessation signals induce these genes. All of our experiments suggest that the *gadd* genes are coordinately regulated: the kinetics of induction for all five transcripts were similar; in addition, overexpression of *gadd* genes was found in homozygous deletion *c<sup>14</sup>CoS/c<sup>14</sup>CoS* mice that are missing a small portion of chromosome 7, suggesting that a *trans*-acting factor encoded by a gene in this deleted portion is a negative effector of the *gadd* genes. The *gadd* genes may represent part of a novel regulatory pathway involved in the negative control of mammalian cell growth.

During typical cell growth, the following phases in the cell cycle have been described: G<sub>1</sub>, S (when DNA synthesis occurs), G<sub>2</sub>, and M (when mitosis occurs). G<sub>0</sub> phase denotes nongrowing cells that have left the cell cycle. There are probably many check-and-balance functions operative throughout the cell cycle and in nongrowing cells, although considerably fewer negative than positive factors in growth control have been identified. For example, in yeast cells there is evidence for regulatory factors that delay entry into the cell cycle from G<sub>0</sub> and block progression at certain points during the cell cycle (discussed in references 18 and 19). Negative growth control elements, such as in retinoblastoma (3), have been identified in mammalian cells. Inhibition of growth in HeLa cells by transfection of DNA from quiescent human fibroblasts also suggests the existence of negative regulatory genes in mammalian cells (20).

Only a limited number of genes that are specifically expressed in response to growth cessation signals have been identified. Six cDNA clones, termed *gas* (growth arrest specific), were recently isolated by hybridization subtraction from mouse 3T3 cells on the basis of preferential expression after growth arrest caused by reduced serum as compared with cells released from growth arrest by serum stimulation (21). It appears for several reasons that the *gas* genes are not coordinately regulated; e.g., their kinetics of induction differ (21). One can conclude from these and other studies that there are multiple genes expressed preferentially in nongrowing cells and that there may be multiple regulatory pathways controlling genes expressed in response to growth arrest.

DNA damage delays progression through the cell cycle and entry into mitosis. In many cases, such delays probably represent active processes and not simply the deleterious effects of the damage. For example, in *Escherichia coli* a role in the inhibition of cell growth is played by the *sulA* gene, a member of the SOS regulon that is induced by DNA damage (18). In yeast cells, the *RAD9* gene product is responsible for the arrest of cells in G<sub>2</sub> produced by X irradiation, and this G<sub>2</sub> arrest is absent in the radiosensitive *rad9* mutant (24). As has been discussed, (18, 24), various check-and-balance functions probably operate elsewhere in the cell cycle or are induced by other types or levels of DNA damage; in particular, the transient inhibition of DNA synthesis seen after DNA damage is decreased or absent in certain DNA-damage-sensitive mutants identified in human, rodent, *Drosophila*, slime mold, and fungal cells.

In this study, we investigated whether the expression of any DNA-damage-inducible (*DDI*) genes is increased by other growth cessation signals. cDNA clones for these *DDI* genes were originally isolated from Chinese hamster ovary (CHO) cells on the basis of rapid induction after UV irradiation (8). We divided the clones into two groups. Class I clones encode transcripts induced only by UV irradiation and UV-mimetic agents, whereas class II clones encode transcripts induced in addition by the alkylating agent methyl methanesulfonate (MMS). We subdivided the second group into class IIa and IIb on the basis of induction by MMS of, respectively, less than and greater than 10-fold. In this report, we show that all three of the class IIb plus two of the class IIa clones code for transcripts that are increased in response to a variety of other growth cessation signals. Evidence is presented that these genes are coordinately

\* Corresponding author.

induced by either DNA-damaging agents or certain other growth cessation signals. These genes may represent part of a heretofore unrecognized regulatory pathway involved in the negative control of cell growth.

## MATERIALS AND METHODS

**Cells and cell culture conditions.** CHO (CHO-K1), V79, NIH 3T3, and Hepa-1 cells and the human fibroblast strain AG1522 (Coriell Institute for Medical Research) were grown and treated with chemical agents as previously described (7, 8, 11). The CHO cells had a doubling time of approximately 12 h, and most experiments were performed with  $2 \times 10^7$  cells per 15-cm-diameter dish; at this density, the cells were growing exponentially. To achieve  $G_0$  phase by medium depletion, we grew cells to  $5 \times 10^7$  per dish (saturation density) and incubated them in 50 ml of medium for an additional 2 days without replacing the medium. HL60 cells (provided by L. M. Neckers) were grown in AIM5 medium (GIBCO Laboratories) as previously described (22). To measure relative DNA synthesis, we incubated dibutyryl cyclic AMP (Bu<sub>2</sub>AMP)-treated HL60 cells with [<sup>3</sup>H]thymidine (0.1  $\mu$ Ci/ml) for 1 h, and incorporation (acid-precipitated label) was compared with that of untreated cells.

For cell cycle studies in HeLa cells, the same poly(A) RNA of synchronized cells as was used previously (25) was kindly provided by B. Z. Zmudka and S. H. Wilson. Briefly, the cells were maintained in 2 mM thymidine for 14 h, allowed to grow in the absence of thymidine for 9 h, exposed to a second block for 14 h, and then released from this block. Under these conditions, greater than 90% of the cells were at the  $G_1/S$  boundary (25).

One male and two female  $c^{ch}/c^{14CoS}$  mice were provided by Liane B. Russell (Oak Ridge National Laboratory, Oak Ridge, Tenn.); subsequent breeding has been carried out in the mouse colony of the Laboratory of Developmental Pharmacology, National Institute of Child Health and Human Development. The livers were removed from newborn animals or 16-day embryos. To determine expression in different adult organs, we isolated RNA from heart, brain, lung, liver, kidney, and skeletal muscle of adult male Wistar rats; the RNA from each tissue of six animals was pooled.

**cDNA clones.** The cDNA clones DDIA153, DDIA45, DDIA34, DDIA33, DDIA7, and DDIA15 were isolated from UV-irradiated CHO cells by hybridization subtraction (8) (for brevity, the DDI prefix is henceforth deleted). The  $\beta$ -actin cDNA clone pA2, which contained a 1.1-kilobase insert, was isolated from Chinese hamster V79 cells (9). The full-length clone for A153 was isolated from cDNA libraries synthesized by the method of Okayama and Berg with CHO cell RNA; these libraries (4) were provided by H. Okayama. Double-stranded plasmid clones were sequenced by using Sequenase (U.S. Biochemical Corp.) as recommended by the manufacturer and described previously (8). Multiple synthetic oligonucleotide primers were made for both strands, which were completely sequenced.

**RNA isolation and analysis.** Cells were lysed in situ with guanidine thiocyanate for RNA isolation, and poly(A) RNA was bound to nylon filters for dot blots or size separated in denaturing gels before transfer, as previously described (11). For denaturing gels, we estimated molecular weights by using single-stranded RNA size markers (Bethesda Research Laboratories, Inc.). Tissues were rapidly frozen in liquid nitrogen, homogenized in guanidine thiocyanate, and handled as described above. cDNA that had been excised from its plasmid vector was labeled (6) and used at  $3 \times 10^6$  to  $5 \times$

$10^6$  dpm/ml. Hybridization conditions were as previously described (11); the final, most stringent rinse was with 40 mM phosphate buffer–1% sodium dodecyl sulfate at 65°C for 40 min. We estimated the poly(A) content of all RNA samples by using a labeled polythymidylic acid probe (11); this correction was small and usually varied by less than 25% from the RNA content measured by optical density at 260 nm. Hybridization was quantified by densitometry measurements of autoradiographs. Relative hybridization was determined by normalizing to the result with RNA from untreated cells isolated in the same experiment on the same day (9, 10). Each value in Results represents the mean of four dot blot determinations; the standard deviation usually varied by less than 25% of the mean.

**Nuclear run-on assays.** Nuclear run-on transcription assays were performed as described previously (11), with minor modifications (9, 10).

## RESULTS

**Expression of DDI genes in CHO cells.** Many of our class II cDNA clones code for transcripts that are induced by UV irradiation and the UV-mimetic agent *N*-acetoxy-2-acetylaminofluorene as well as by other DNA-damaging agents such as the alkylating agents MMS and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, the bifunctional agents nitrogen mustard and *cis*-Pt(II) diamminedichloride, and H<sub>2</sub>O<sub>2</sub> (8). Our three class IIb transcripts are particularly highly induced by these agents, all of which are known to inhibit DNA synthesis and cell growth.

It is possible that transduction of a common signal, elicited by both DNA damage and growth cessation signals, will lead to the induction of genes coordinately involved in the response to these two adverse stimuli. We therefore examined the levels of these transcripts in growth-arrested CHO cells. The three class IIb and three class IIa transcripts shown in Fig. 1 were all induced by MMS. These cDNA probes hybridized to different-size transcripts, and sequence analysis (see below) confirmed that each cDNA was different and corresponded to nothing in the current GenBank data base. It should be noted that all of these transcripts were expressed, albeit at lower levels, in untreated growing cells and could be visualized with longer autoradiograph exposure times. The class IIb transcripts were markedly induced by growth arrest caused either by medium depletion or serum reduction (Fig. 1 and Table 1); both of these treatments caused cells to accumulate in  $G_0$  phase. In addition, two class IIa transcripts, A33 and A7, were found to be increased after growth cessation. These five cDNA clones differed from A15 and our other class II and the class I clones, which showed no evidence for induction by growth cessation. For example, we found that A8, A9, A18, A29, A31, A50, A70, A77, A90, A94, A99, and A115 transcripts were not induced by medium depletion or reduced serum (data not shown). No evidence for induction in response to growth cessation has been found for a variety of other genes induced by DNA damage or other types of stress; these genes include  $\beta$ -polymerase (12, 25), ubiquitin (10), *c-fos* (15), and more than 10 different heat-shock-inducible genes (9).  $\beta$ -Actin mRNA, which served as a control, was not induced but instead was consistently decreased in  $G_0$  cells, as has been reported by others for various actin transcripts (21). It should be emphasized that the yields of poly(A) RNA per cell were similar in untreated, MMS-treated, and  $G_0$  cells and have been published previously for some of the samples used in this study (12, 25). A153, A45, A34, A33, and A7 therefore represent a

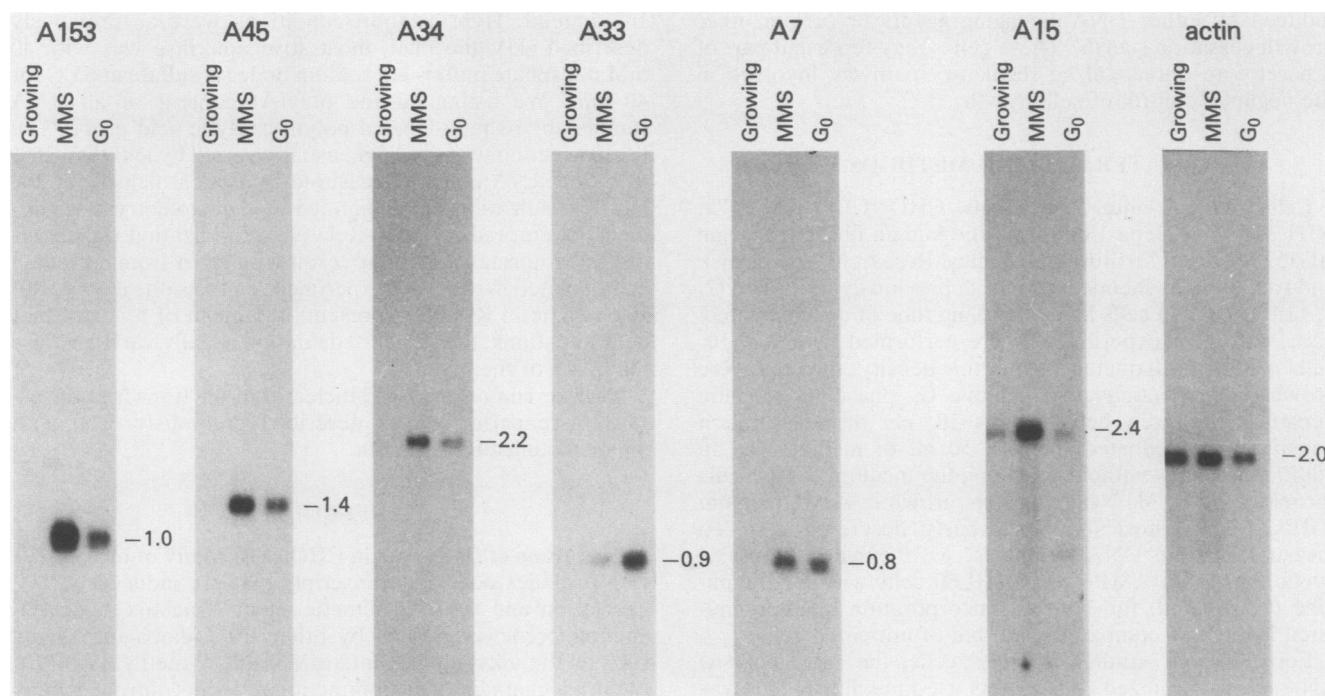


FIG. 1. Northern (RNA) hybridization analysis of RNA from growing, MMS-treated, and nongrowing  $G_0$  CHO cells. Equal amounts of poly(A) RNA were size separated and hybridized with the indicated labeled cDNA probe; pA2 was used to detect  $\beta$ -actin mRNA. A153, A45, and A34 are class IIb clones; A33, A7, and A15 are class IIa clones. Exponentially growing cells were treated with MMS (100  $\mu$ g/ml) for 4 h.  $G_0$  cells were grown to high density ( $5 \times 10^7$ /15-cm-diameter dish) and incubated for 2 days without refeeding. Values to the right of each lane indicate the estimated size (in kilobases) of the hybridizing transcript.

subset of stress-induced genes that are induced in response to certain growth cessation signals and have been termed *gadd* (growth arrest and DNA damage inducible).

Response of the *gadd* genes was examined after various cell treatments (Table 1). Even though the cells are still viable under our medium depletion conditions, the levels of

TABLE 1. Fold induction of RNA levels in CHO cells after exposure to DNA-damaging agents or growth arrest signals

Treatment	RNA level with given cDNA probe <sup>a</sup>						
	A153	A45	A34	A33	A7	A15	$\beta$ -Actin
MMS (100 $\mu$ g/ml, 4 h) <sup>b</sup>	35.0	19.0	20.0	5.6	6.4	5.5	1.3
Medium depletion <sup>c</sup>	17.0	14.0	6.1	20.0	3.7	0.7	0.4
Reduced serum <sup>d</sup>	13.0	9.7	5.0	2.4	3.4	1.3	0.6
Hydroxyurea (2 mM, 24 h) <sup>b</sup>	9.1	7.5	6.1	9.0	3.0	1.6	0.5
Hydroxyurea (2 mM, 4 h) <sup>b</sup>	1.2	1.4	1.5	0.9	0.8	1.3	1.0
Ara-C (10 $\mu$ M, 4 h) <sup>b</sup>	0.8	1.0	1.2	1.3	0.6	1.0	1.1
Hydroxyurea + ara-C <sup>e</sup>	1.7	1.0	1.5	1.3	1.0	1.3	1.2
TPA (400 ng/ml, 4 h) <sup>f</sup>	1.3	1.1	1.2	0.9	0.8	1.2	1.1

<sup>a</sup> The relative abundance of RNA from treated samples was determined by RNA dot blot hybridization and normalized to that of untreated, exponentially growing cells isolated at the same time.

<sup>b</sup> Exponentially growing cells were treated with chemical agents for the indicated times and harvested.

<sup>c</sup> Cells were grown to high density ( $5 \times 10^7$ /15-cm-diameter dish) and incubated for 2 days without medium change.

<sup>d</sup> Exponentially growing cells were incubated in medium containing 0.5% serum for 24 h.

<sup>e</sup> Treatment combined the same concentrations and exposure time, 4h, as above.

<sup>f</sup> No induction was seen after a 4-h exposure over a range from 20 to 1,600 ng of TPA per ml.

many nutrients and metabolites are altered compared with those in growing cells. We therefore compared the effect of reduction of serum concentration with that of medium depletion, and the induction of all five transcripts was found to be similar to that seen during medium depletion. In all experiments, induction, as measured by the relative level of RNA compared with that in untreated cells, was greater after MMS treatment than after either type of growth cessation treatment not involving DNA damage. The exception was A33 RNA, for which the magnitude of induction was much more variable than that for the other four transcripts; e.g., induction of A33 RNA by MMS varied from 4.9- to 21-fold in six experiments done on different days (not shown). No induction of any of the *gadd* genes was seen by treatment with the tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), which is known to induce certain other DNA-damage-inducible genes, or by other stress signals such as heat shock (1, 8).

Nuclear run-on experiments indicated that the induction of the *gadd* genes in response to both MMS treatment and growth arrest was due to an increased rate of transcription (Fig. 2 and Table 2). In comparison, A15 transcription was increased only after MMS treatment, not in  $G_0$  (Fig. 2); Table 2 shows the results for other cDNA clones that encode transcripts not increased in nongrowing cells. In both Fig. 1 and 2, the autoradiograph exposure time was much shorter for  $\beta$ -actin, suggesting that the other transcripts were much less abundant. We estimated that the frequency of A153 or A45 clones in cDNA libraries constructed from untreated growing CHO cells or human fibroblasts is substantially less than 0.01%. For all five *gadd* clones, Southern blot analysis of genomic DNA was consistent with a single different locus for each (data not shown). Thus, induction appears to

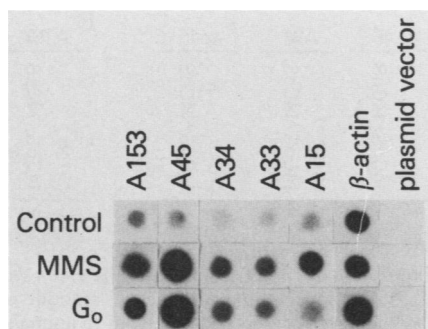


FIG. 2. Nuclear run-on transcription assay. Replicate DNA dot blots were hybridized with labeled RNA synthesized from isolated nuclei (see Materials and Methods). Nuclei from control untreated growing cells, cells treated with MMS (100  $\mu$ g/ml) for 2 h, or nongrowing cells in depleted medium (see Table 1) ( $G_0$ ) were used as indicated. The plasmid DNA used is indicated above each lane. The autoradiograph exposure time was identical for the three dot blots of each lane but varied between lanes. Results with the fifth *gadd* clone, A7, are not included, since the hybridization signal was weak and induction could not be convincingly demonstrated.

represent an increased rate of transcription for these different single-copy genes (see Discussion).

**Kinetics of induction for the *gadd* transcripts.** We next investigated the time course and dose response for MMS induction (Fig. 3). Induction of these transcripts was rapid during the 4-h MMS exposure (Fig. 3A). Although results are shown only for A153, the induction of all five *gadd* transcripts was blocked by actinomycin D, which supports the data shown in Fig. 2 indicating that induction was primarily due to increased transcription. After MMS removal, transcript levels decreased at various rates and reached untreated levels by 25 h except for A33; this observation, and results of other studies to be presented elsewhere, suggest that the A33 transcript is more stable than the other transcripts. In the dose-response experiment (Fig. 3B), all five transcripts exhibited maximal induction with the same dose. In general, the similar kinetics for induction of all five transcripts are consistent with coordinate induction.

We found that growth cessation signals other than treatment with DNA-damaging agents slowly induced all five *gadd* transcripts. Incubation of growing cells in medium containing either 0.5% or no serum consistently resulted in no induction during the first 8 h and maximal induction only after 24 h or longer (not shown). Since there is probably a lag time after removal of the serum and depletion of the cellular stores of serum factors necessary for growth, we used an

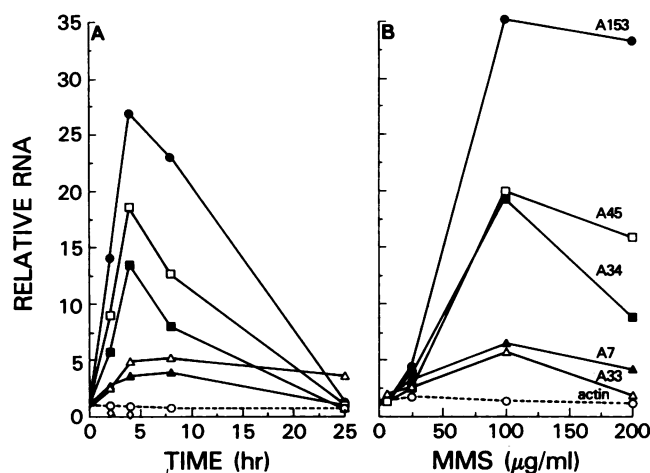


FIG. 3. Time course (A) and dose response (B) for induction of the *gadd* transcripts in CHO cells by MMS. The relative level of RNA of treated samples compared with untreated controls was determined as in Table 1. In panel A, MMS (100  $\mu$ g/ml) was added at the start of the experiment and removed after 2 h for the earliest time point and after 4 h for the other time points. For later time points, a portion of the same medium that had been removed before the addition of MMS was added back to the cells. In the experiment of panel B, which was done on a different day, cells were exposed to the indicated dose of MMS for 4 h. Symbols are the same in both panels with the exception that values designated  $\diamond$  in panel A represent A153 RNA after the addition of actinomycin D (5  $\mu$ g/ml) 10 min before the addition of MMS.

alternate approach of employing DNA synthesis inhibitors to produce growth cessation more rapidly (Table 1). These DNA synthesis inhibitors are known to block DNA synthesis within minutes of addition. The *gadd* transcripts were not induced in response to a 1- to 8-h exposure to hydroxyurea, cytosine arabinoside (ara-C), or a combination of hydroxyurea and ara-C (Table 1). On the other hand, after a 24 h exposure to hydroxyurea, all five *gadd* transcripts were induced concurrently; these results were very similar to that produced by serum reduction or medium depletion (Table 1). Thus, induction was not due to the inhibition of DNA synthesis per se but was produced after a prolonged (24-h) inhibition of cell growth by hydroxyurea. We found that release from growth arrest led to a decrease in the level of these transcripts comparable to that seen after removal from MMS (not shown); e.g., in two separate experiments with A153 RNA induced by serum reduction, 90 and 86%, respectively, of the increase was lost after a 4-h incubation in medium containing 10% serum (serum stimulation).

**Expression of *gadd* transcripts throughout the cell cycle and in nongrowing cells after terminal differentiation.** Both A153 and A45 cDNA hybridize at high stringency to a corresponding transcript in human cells, whereas A34, A33, and A7 do not (8). We were therefore able to conduct a series of experiments with A153 and A45 in human cells (summarized in Table 3). For example, we determined the level of these transcripts in HeLa cells synchronized by a double thymidine block, which arrests cells near the boundary between  $G_1$  and S phases. During both the thymidine block and throughout the cell cycle after release, the relative levels of the A153 and A45 transcripts varied by less than 50%. We also achieved growth arrest conditions by inducing the differentiation of HL60 cells. Exposure to Bu<sub>2</sub>AMP or TPA has been shown to induce terminal monocytic differentiation of this promyelocytic cell line (22). Interestingly, treatment

TABLE 2. Densitometric measurements of nuclear run-on experiment from Fig. 2

Cells	Measurement with given plasmid DNA <sup>a</sup>								
	A153	A45	A34	A33	$\beta$ -Actin	A15	A94 <sup>b</sup>	A121 <sup>b</sup>	A140 <sup>b</sup>
MMS treated	4.6	16.0	6.7	4.3	0.8	5.4	2.0	2.3	1.8
$G_0$	3.5	13.6	4.8	2.9	1.9	1.2	1.6	1.3	1.2

<sup>a</sup> Values represent results obtained from either MMS-treated or nongrowing cells normalized to that of control (untreated growing) cells; the values were derived by dividing the average of two dot blot determinations for treated samples by the average of two for control samples. Labeled RNA was hybridized to various plasmids bound to filters as described in the legend to Figure 2.

<sup>b</sup> Data not shown in Fig. 2 for a class IIa cDNA clone that encodes transcripts that are not increased in nongrowing cells.

TABLE 3. Expression of A153 mRNA in other mammalian cells

Cell type	Expression <sup>a</sup>	
	Increased	Unchanged
Human		
HeLa	MMS <sup>b</sup> (++) Medium depletion <sup>d</sup> (++)	Throughout cell cycle <sup>c</sup> G <sub>1</sub> /S thymidine block <sup>c</sup>
HL60	MMS (++)	Growth cessation by differentiation
Skin fibroblasts	MMS (++) Contact inhibition <sup>c</sup> (+)	
Chinese hamster V79 lung fibroblasts	MMS (+)	
Mouse		
3T3	Contact inhibition (++)	
Hepa-1	MMS (++)	

<sup>a</sup> The relative level of A153 mRNA was compared with that of untreated growing cells as described in Table 1, footnote a. +, Induction of 3- to 10-fold; ++, induction of 10- to 20-fold.

<sup>b</sup> In all cases, the MMS exposure was the same as that in Table 1. Rapid induction was seen only with MMS and other DNA-damaging agents such as H<sub>2</sub>O<sub>2</sub>, cis-Pt(II) diamminedichloride, nitrogen mustard, N-acetoxy-2-acetylaminofluorene, N-methyl-N'-nitro-N-nitrosoguanidine, UV radiation (254 nm), and near-UV radiation (320 nm).

<sup>c</sup> Cells were arrested at the G<sub>1</sub>/S boundary with a double thymidine block and then released by incubation in fresh medium.

<sup>d</sup> See Table 1, footnote c.

<sup>e</sup> Cells were grown to stationary contact-inhibited cultures and refed with fresh medium 1 day before harvest.

with Bu<sub>2</sub>AMP induced morphologic changes characteristic of differentiation, cessation of cell growth, and a marked decrease in DNA synthesis but induced no increase in A153 or A45 RNA (Fig. 4). The same results were obtained after TPA exposure (not shown). These cells, as well as all other mammalian cells examined (Table 3), exhibited marked induction of these transcripts by MMS.

**Overexpression of three *gadd* transcripts in *c*<sup>14CoS</sup>/*c*<sup>14CoS</sup> mice.** Several laboratories have studied inbred mouse lines with radiation-induced chromosomal deletions involving the albino locus (*c*) on chromosome 7 and found indirect evidence for one or more regulatory genes located within the

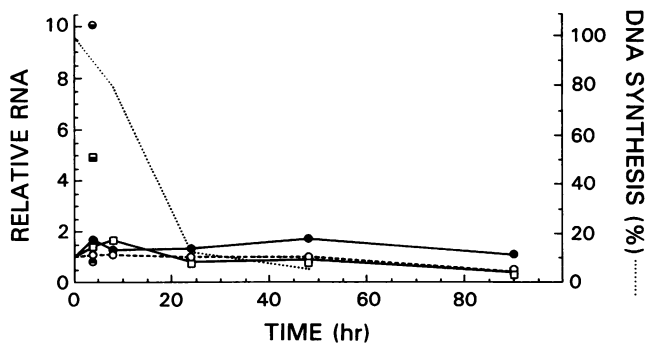


FIG. 4. Expression of *gadd* transcripts in HL60 cells. Bu<sub>2</sub>AMP was added to growing cells, and relative RNA abundance was determined as described in Table 1 at the indicated times after addition; symbols are as in Fig. 3. Symbols at the 4-h time point indicate samples treated with MMS (100 μg/ml) rather than Bu<sub>2</sub>AMP: ●, A153; ■, A45, and ○, β-actin. The dotted line represents percent DNA synthesis after Bu<sub>2</sub>AMP addition relative to that in untreated growing cells.

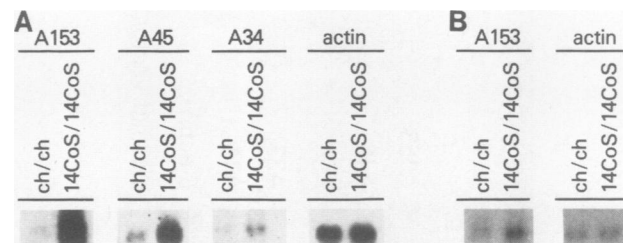


FIG. 5. Expression of *gadd* transcripts in the *ch/ch* wild type and the *c*<sup>14CoS</sup>/*c*<sup>14CoS</sup> deletion homozygote. Equal amounts of poly(A)-enriched RNA from mouse livers (pooled from 15 animals) were size separated and hybridized with the indicated cDNA probe as described in the legend to Fig. 1; only the hybridizing band is shown. Results with the β-actin probe are included as a control. The source of the samples was poly(A) RNA obtained from newborn liver (A) or whole-cell RNA obtained from 16-day embryos (B).

missing region of *c*<sup>14CoS</sup>/*c*<sup>14CoS</sup> mice (13, 14). The NAD(P)H: menadiadione oxidoreductase (*NMO1*) gene is known to encode a quinone reductase believed to play a central role in protection against oxidative stress (5). The rate of *NMO1* gene transcription was recently found to be markedly higher in the *c*<sup>14CoS</sup>/*c*<sup>14CoS</sup> deletion homozygote than in the *c*<sup>ch</sup>/*c*<sup>ch</sup> wild type and the *c*<sup>ch</sup>/*c*<sup>14CoS</sup> heterozygote (D. D. Petersen, F. J. Gonzalez, C. A. Kozak, V. Rapic, J.-Y. Lee, J. E. Jones, and D. W. Nebert, Proc. Natl. Acad. Sci. USA, in press). Many quinones and other oxidative metabolites that induce *NMO1* are known to break DNA and transiently inhibit DNA synthesis and cellular growth (5). The *NMO1* enzyme would be advantageous to the cell by metabolizing quinones, thereby protecting against DNA damage. In addition, we have recently found that *NMO1* mRNA is induced after a 4-h exposure to MMS in mouse Hepa-1 cells (unpublished results). We therefore considered the possibility of a common regulatory pathway between the DNA-damage-inducible genes and the *NMO1* gene.

We compared expression of the various DNA-damage-inducible genes in *c*<sup>14CoS</sup>/*c*<sup>14CoS</sup> and *c*<sup>ch</sup>/*c*<sup>ch</sup> newborn livers. Interestingly, levels of the A153, A45, and A34 transcripts were markedly higher in *c*<sup>14CoS</sup>/*c*<sup>14CoS</sup> than in *c*<sup>ch</sup>/*c*<sup>ch</sup> mice (Fig. 5). The autoradiograph exposure times in Fig. 5 were chosen to demonstrate that these transcripts were present in both *c*<sup>ch</sup>/*c*<sup>ch</sup> and *c*<sup>14CoS</sup>/*c*<sup>14CoS</sup> RNAs, in addition to showing the marked increase in the deletion homozygote mutant. Even with exposure times of 4 weeks, however, no hybridization to A33, A7, A8, A31, and A99 probes could be detected. These data may indicate that these genes are not expressed in newborn liver. We also found that the expression of other *DDI* transcripts, such as A9, A15, A29, A50, A70, A77, A88, A90, A94, A108, A109, A115, A143, A144, A162, U1 (metallothionein II), U2 (metallothionein I), and β-actin RNAs, were not increased in the *c*<sup>14CoS</sup>/*c*<sup>14CoS</sup> mice. These results provide additional evidence for the coordinate regulation of the A153, A45, and A34 genes. Elevation of the A153 mRNA could be detected in the 16-day-old *c*<sup>14CoS</sup>/*c*<sup>14CoS</sup> fetal liver. These results suggest that overexpression of the A153 gene is not related to death of *c*<sup>14CoS</sup>/*c*<sup>14CoS</sup> mice in the newborn period.

Additional *in vivo* studies were undertaken with A153 cDNA, which hybridizes strongly at high stringency to a 1-kilobase transcript in rodent cells and tissues. The A153 transcript was detectable in all rat tissues examined: brain, heart, liver, kidney, lung, spleen, and skeletal muscle (data not shown). The relative level was similar in all tissues

```

80
AGTAGCGTGAGTCTCACACTTCGGTTATATCATGTTGAAGATGAACGGGTGGCAGCGACAGAGCCAAAATAAAAGCCGGG
160
ACCTGAGGAGCGAGTGTTCAGAAAGGAAGTGTATCTTCATACATCACCATACCTGAAAGCAGAACCCAGTCCAACCTACAG
219
AC  ATG GCA GCT GAG TCC CTG CCA TTC ACC TTG GAG ACG GTG TCC AGC TGG GAG CTG GAA
    Met Ala Ala Glu Ser Leu Pro Phe Thr Leu Glu Thr Val Ser Ser Trp Glu Leu Glu
279
GCG TGG TAT GAG GAT CTG CAG GAG GTG CTG TCC TCG GAT GAA AAT GGG GGT CCC TAT AGC
    Ala Trp Tyr Glu Asp Leu Gln Glu Val Leu Ser Ser Asp Glu Asn Gly Gly Pro Tyr Ser
339
TCA TCC CTT GGA AAC GAA GAG GGG GAG TCA AAA ACC TTC ACT ACC CTA GAC CCT GCA TCC
    Ser Ser Leu Gly Asn Glu Glu Gly Glu Ser Lys Thr Phe Thr Thr Leu Asp Pro Ala Ser
399
CTA GCT TGG CTG ACT GAG GAG CCG GGG CCA GCA GAG GTC ACA AGC AGC TCC CAG AGC CCC
    Leu Ala Trp Leu Thr Glu Glu Pro Gly Pro Ala Glu Val Thr Ser Ser Ser Gln Ser Pro
459
CGC TCT CCA GAT TCC AGT CAG AGC TGT ATG GCT CAG GAG GAA GAG GAA GAT CAA GGA CGA
    Arg Ser Pro Asp Ser Ser Gln Ser Cys Met Ala Gln Glu Glu Glu Glu Asp Gln Gly Arg
519
ACC AGG AAA CGG AAA CAG AGT GGT CAG TGC CCA GCC CGG GGC ACT GGG AAG CAG CGC ATG
    Thr Arg Lys Arg Lys Ser Gly Gln Cys Pro Ala Arg Gly Thr Gly Lys Gln Arg Met
579
AAG GAG AAA GAA CAG GAG AAC GAG AGG AAA GTG GCT CAG CTA GCT GAG GAG AAC GAG CGG
    Lys Glu Lys Glu Gln Glu Asn Glu Arg Lys Val Ala Gln Leu Ala Glu Glu Asn Glu Arg
639
CTC AAG CAG GAA ATC GAG CGC CTG ACC AGA GAG GTA GAG GCC ACT CGT CCG GGC TCT GAT
    Leu Lys Gln Glu Ile Glu Arg Leu Thr Arg Glu Val Glu Ala Thr Arg Pro Gly Ser Asp
710
CGA CCG CAT GTA AAT CTG CAA CAA GTA TGAACAATTGGGACTATCATTCGCCAACTTGAGTGACCTCCTGT
    Arg Pro His Val Asn Leu Gln Gln Val
790
CTCTCCTGGACCATCATGTCAATGCCAAGGATGCTGGGTAGGGAGAACAAAGGTCTCAGCTAGTGTGTATATATTGTA
837
CATTCTTTATTATTTATTGTCTGTACCCGGTAAAGTGACTTTGTAT

```

FIG. 6. DNA and predicted protein sequences (unmodified molecular weight, 18,811) from our putative full-length A153 cDNA clone.

except for brain and heart, in which the level of A153 was severalfold higher.

**Sequence analysis.** We determined the DNA sequences for all five *gadd* cDNA clones, including a full-length clone of A153 (Fig. 6). This 837-nucleotide sequence, excluding the poly(A) tail, contains one large open reading frame from positions 73 to 666, which encodes a protein of 168 amino acids ( $M_r$ , 18,811) from an ATG translation start site at position 163. Neither A153 nor the other cDNA clones (not shown) correspond to known sequences (GenBank, release 58.0).

The predicted amino acid sequence of A153 does not exhibit significant similarity to other known sequences (NBRF, release 18.0). The protein is generally unremarkable except that it has a significant hydrophobic moment; i.e., one end is very different in charge from the other end. This type of structure suggests that the carboxy terminus might be important in touching a membrane or cellular matrix or in contacting another cellular protein.

## DISCUSSION

DNA damage transiently inhibits DNA synthesis and cell growth. In this study, the five *gadd* genes were found to be rapidly induced by DNA-damaging agents as well as by growth arrest in cells that have left the cell cycle and entered  $G_0$ . Evidence for identical responses was found in other mammalian cells, including established cell lines and in transformed human fibroblasts. Our data with cells other than CHO are summarized in Table 3 for A153, which was the most strongly induced transcript in CHO cells and for which the entire sequence has been determined; however, the same pattern of induction was observed for A45. A series of experimental conditions that caused growing cells to leave the cell cycle and enter  $G_0$  caused induction of A153 RNA (Tables 1 and 3). In all cell types, A153 mRNA was always rapidly and strongly induced by MMS (and other DNA-

damaging agents), whereas induction by growth arrest was slower and never exceeded that for MMS in the same experiment. This effect occurred both in transformed cells, such as CHO and HeLa cells, and in nontransformed cells, such as 3T3 cells and human fibroblasts, which enter a defined  $G_0$  phase between mitosis and S phase after contact inhibition. By fluorescence-activated cell sorting analysis of DNA content, more than 90% of CHO cells that were treated with reduced serum or by medium depletion were also found to have accumulated in  $G_1$ . Induction of A153 RNA presumably occurs only in growing cells that have been forced into  $G_0$  but still have the capacity for growth. For example, growth arrest by contact inhibition, serum reduction, or medium depletion can be quickly reversed by replating at lower density or by adding complete medium. In contrast, no induction occurred in HL60 cells that had lost the capacity to grow after terminal differentiation; in addition, the level of A153 RNA was not elevated in differentiated tissues *in vivo*. Unlike the other treatments that induce these transcripts in  $G_0$ , DNA damage causes a rapid transient inhibition of DNA synthesis and growth throughout the cell cycle. This rapid induction in growing cells appears to be quite specific for DNA-damaging agents, because a variety of other stresses and inhibitors do not produce this effect. Considering the pattern of induction for A153 and the other four *gadd* genes, we believe that these genes may play a role in the inhibition of growth after DNA damage as well as in the maintenance of growth arrest in  $G_0$ .

Results of nuclear run-on experiments (Fig. 2 and Table 2) indicate that induction of the *gadd* genes primarily reflects increased transcription but do not preclude the possibility that some of the induction represents increased mRNA stability. In particular, the fold increase in nuclear run-on transcriptional rates for A153, A34, and A33 in MMS-treated or  $G_0$  cells shown in Table 2 is less than the fold increase in RNA levels shown in Table 1. For the run-on experiment

with  $G_0$  cells, the values for these *gadd* genes were 2.9- to 4.8-fold, whereas those for  $\beta$ -actin and other noninducible genes were 1.2- to 1.9-fold; in the same experiment, the value with  $G_0$  cells for A18, a class I *DDI* gene whose mRNA level is constant throughout the cell cycle and in  $G_0$  (M. Papathanasiou, M. C. Hollander, and A. J. Fornace, Jr., manuscript in preparation), was found to be 1.2-fold. However, transcription for these low-abundance RNA species has probably been underestimated in the nuclear run-on experiment because of the weak signals and background hybridization. In contrast to the plasmid vector with which there was little hybridization, the cDNA clones contain GC tails, which in our experience increase nonspecific hybridization; in addition, low levels of cross-hybridization to other sequences may contribute to the background. These types of experimental difficulties must be kept in mind for low-abundance transcripts when the sequence being studied probably represents <0.001% of the total RNA probe. In other nuclear run-on experiments, the difference between A153 and A45 was less than that shown in Table 2; e.g., in one experiment, transcription as measured by nuclear run-on was increased 9- and 11-fold, respectively, for A153 and A45 in MMS-treated cells. To address the question of increased mRNA stability, we carried out preliminary experiments with RNA synthesis inhibitors (data not shown). A33 RNA appeared to be very stable in both untreated and MMS-treated cells, since no appreciable decrease was detected after a 4-h exposure to actinomycin D. In both untreated and MMS-treated cells, actinomycin D experiments indicated that A153 RNA has a short half-life, similar to the results described earlier.

Our *gadd* cDNA clones have several similarities to, but also differences from, other transcripts and proteins expressed specifically in growth-arrested cells. Like the *gas* genes (21) and statin, a nuclear protein found in human cells (23), the *gadd* genes are induced in  $G_0$  cells substantially after growth cessation, and their expression decreases rapidly after the restriction to growth has been removed. Unlike these examples, however, the *gadd* genes are clearly expressed in growing cells. The kinetics of induction indicate that the *gas* genes are not coordinately regulated (21), whereas the *gadd* genes are. The pattern of expression in rodent tissues in vivo for *gas1* and *gas2* RNAs is different from that for A153. None of the *gadd* cDNA sequences shows any homology to the *gas2* sequence. In another published report, an abundant 20-kilodalton protein has been found in quiescent chicken heart cells but not in growing cells or in transformed cells even at high density (2). In contrast, the *gadd* transcripts are not abundant yet are expressed in both untransformed and established cell lines that exhibit inducible expression. There are probably multiple regulatory pathways that inhibit or delay cell growth. Our clones were originally isolated on the basis of rapid induction by DNA-damaging agents, whereas these other examples were clones selected on the basis of expression in growth-arrested cells. Hence, it would not be unexpected that regulation of the *gadd* genes differs from that of other growth cessation genes.

A variety of experiments indicate that these five *gadd* genes are coordinately regulated. The kinetics of induction in CHO cells are similar for all five genes. In other cell types, including human and mouse, the patterns of induction are very similar for A153 and A45. Studies with the other clones were inconclusive, probably because their sequences are not as highly conserved. For example, A153 and A45 hybridized at high stringency with human genomic DNA, whereas

specific hybridization could not be detected with A34, A33, and A7 under the same conditions. The elevated expression of A153, A45, and A34 mRNAs in  $c^{14CoS}/c^{14CoS}$  deletion homozygote mice provides additional evidence for coordinate regulation. Considering the multiple examples of coordinate regulation, we suggest that these five *gadd* genes could very well represent members of the same regulon, which is activated by DNA damage or other growth arrest signals; moreover, this regulon may be involved in negative growth control.

There are several possible mechanisms for the overexpression seen in the  $c^{14CoS}/c^{14CoS}$  deletion homozygotes. One possibility is that overexpression of A153, A45, and A34 is a secondary effect. For example, these animals are known to develop hypoglycemia (14) and generally develop necrosis of the bowel and die within 18 h after birth. One might argue that the hepatic cells have stopped growing as the newborn is about to die; this tenet is not likely, however, in view of the fact that the A153 transcript is overexpressed in the  $c^{14CoS}/c^{14CoS}$  fetus at gestational day 16, as compared with the 16-day-old  $c^{ch}/c^{ch}$  fetus (Fig. 5). The more likely possibility, therefore, is that overexpression is due to the loss of a specific negative regulatory factor encoded by a gene located in the deleted portion of chromosome 7. Overexpression of the *NMO1* gene in the  $c^{14CoS}/c^{14CoS}$  fetus is easily detected at gestational day 16 (Petersen et al., in press). This finding provides strong evidence that *NMO1* gene activation is also not correlated with neonatal death. The deleted chromosomal region in  $c^{14CoS}/c^{14CoS}$  mice has been estimated to be about 1.2 centimorgans long (13). It has been estimated that about 30 genes would be present in a region of 1 centimorgan of genomic DNA (17). We would therefore predict that the  $c^{14CoS}/c^{14CoS}$  deletion might comprise less than three dozen genes. The increased expression of the *NMO1* gene and the *gadd* genes that are expressed in newborn liver in the deletion homozygote as compared with the wild type suggests that a gene on chromosome 7 may encode one or more *trans*-acting factors that would be negative effectors of the *NMO1* and *gadd* genes. In any event, these interesting results provide additional evidence for the coordinate regulation of the *gadd* genes.

#### ACKNOWLEDGMENTS

We thank Timothy Kinsella (University of Wisconsin, Madison) for his support and encouragement of this work, George Michaels and Kathleen Dixon (National Institute of Child Health and Human Development, Bethesda, Md.) for valuable discussions, Leonard Neckers (National Cancer Institute, Bethesda, Md.) for fluorescence-activated cell sorting analysis, and Robert Jernigan (National Cancer Institute, Bethesda, Md.) for support in DNA sequence analysis.

#### LITERATURE CITED

1. Angel, P., A. Pöting, U. Mallick, H. J. Rahmsdorf, M. Schorpp, and P. Herrlich. 1986. Induction of metallothionein and other mRNA species by carcinogens and tumor promoters in primary human skin fibroblasts. *Mol. Cell. Biol.* 6:1760-1766.
2. Bedard, P. A., S. D. Balk, H. S. Gunther, A. Morisi, and R. L. Erikson. 1987. Repression of quiescence-specific polypeptides in chicken heart mesenchymal cells transformed by rous sarcoma virus. *Mol. Cell. Biol.* 7:1450-1458.
3. Cavanee, W. K., T. P. Dryja, R. A. Phillips, W. F. Benedict, R. Godbout, B. L. Gallie, A. L. Murphree, L. C. Strong, and R. L. White. 1983. Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. *Nature (London)* 305:779-784.
4. Chin, D. J., G. Gil, D. W. Russell, L. Liscum, K. L. Luskey, S. K. Basu, H. Okayama, P. Berg, J. L. Goldstein, and M. S. Brown. 1984. Nucleotide sequence of 3-hydroxy-3-methyl-glu-

- tary coenzyme A reductase, a glycoprotein of endoplasmic reticulum. *Nature (London)* **308**:613-617.
5. **Ernster, L., R. W. Estabrook, P. Hochstein, and S. Orrenius (ed).** 1987. DT diaphorase: a quinone reductase with special functions in cell metabolism and detoxication. Cambridge University Press, Cambridge.
  6. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**:6-13.
  7. **Fornace, A. J., Jr.** 1983. Recombination of parent and daughter strand DNA after UV-irradiation in mammalian cells. *Nature (London)* **304**:552-554.
  8. **Fornace, A. J., Jr., I. Alamo, Jr., and M. C. Hollander.** 1988. DNA damage-inducible transcripts in mammalian cells. *Proc. Natl. Acad. Sci. USA* **85**:8800-8804.
  9. **Fornace, A. J., Jr., I. Alamo, Jr., and M. C. Hollander.** 1989. Induction of heat shock protein transcripts and B2 transcripts by various stresses in Chinese hamster cells. *Exp. Cell Res.* **182**:61-74.
  10. **Fornace, A. J., Jr., I. Alamo, Jr., M. C. Hollander, and E. Lamoreaux.** 1989. Ubiquitin RNA is a major stress-induced transcript in mammalian cells. *Nucleic Acids Res.* **17**:1215-1230.
  11. **Fornace, A. J., Jr., and J. B. Mitchell.** 1986. Induction of B2 RNA polymerase III transcription by heat shock; enrichment for heat shock induced sequences in rodent cells by hybridization subtraction. *Nucleic Acids Res.* **14**:5793-5811.
  12. **Fornace, A. J., Jr., B. Z. Zmudka, M. C. Hollander, and S. H. Wilson.** 1989. Induction of mammalian  $\beta$ -polymerase mRNA by DNA damaging agents in Chinese hamster ovary cells. *Mol. Cell. Biol.* **9**:851-853.
  13. **Gluecksohn-Waelsch, S.** 1979. Genetic control of morphogenetic and biochemical differentiation: lethal albino deletions in the mouse. *Cell* **16**:225-237.
  14. **Gluecksohn-Waelsch, S.** 1987. Regulatory genes in development. *Trends Genet.* **3**:123-127.
  15. **Hollander, M. C., and A. J. Fornace, Jr.** 1989. Induction of *fos* RNA by DNA damaging agents. *Cancer Res.* **49**:1687-1692.
  16. **Huisman, O., and R. D'Ari.** 1981. An inducible DNA replication-cell division coupling mechanism in *E. coli*. *Nature (London)* **290**:797-799.
  17. **Landegren, U., R. Kaiser, C. T. Caskey, and L. Hood.** 1988. DNA diagnostics—molecular techniques and automation. *Science* **242**:229-237.
  18. **Lavin, M. F., and A. L. Schroeder.** 1988. Damage-resistant DNA synthesis in eukaryotes. *Mutat. Res.* **193**:193-206.
  19. **Lee, M. G., C. J. Norbury, N. K. Spurr, and P. Nurse.** 1988. Regulated expression and phosphorylation of a possible mammalian cell-cycle control protein. *Nature (London)* **333**:676-679.
  20. **Padmanabhan, R., T. H. Howard, and B. H. Howard.** 1987. Specific growth inhibitory sequences in genomic DNA from quiescent human embryo fibroblasts. *Mol. Cell. Biol.* **7**:1894-1899.
  21. **Schneider, C., R. M. King, and L. Philipson.** 1988. Genes specifically expressed at growth arrest of mammalian cells. *Cell* **54**:787-793.
  22. **Trepel, J. B., O. R. Colamonici, K. Kelly, G. Schwab, R. A. Watt, E. A. Sausville, E. S. Jaffe, and L. M. Neckers.** 1987. Transcriptional inactivation of *c-myc* and the transferrin receptor in dibutyryl cyclic AMP-treated HL-60 cells. *Mol. Cell. Biol.* **7**:2644-2648.
  23. **Wang, E.** 1987. Contact-inhibition-induced quiescent state is marked by intense nuclear expression of *statin*. *J. Cell. Physiol.* **133**:151-157.
  24. **Weinert, T. A., and L. H. Hartwell.** 1988. The *RAD9* gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. *Science* **241**:317-322.
  25. **Zmudka, B. Z., A. Fornace, J. Collins, and S. H. Wilson.** 1988. Characterization of DNA polymerase  $\beta$  mRNA: cell-cycle and growth response in culture human cells. *Nucleic Acids Res.* **16**:9587-9596.